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Metagenome-Derived Clones Encoding Two Novel Lactonase Family Proteins Involved in Biofilm Inhibition in *Pseudomonas aeruginosa*▽†

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Here we report the isolation and characterization of three metagenome-derived clones that interfere with bacterial quorum sensing and degrade *N*-(3-oxooctanoyl)-L-homoserine lactone (3-oxo-C_8-HSL). By using a *traI-lacZ* gene fusion, the metagenome-derived clones were identified from a soil DNA library and analyzed. The open reading frames linked to the 3-oxo-C_8-HSL-degrading activities were designated bpiB01, bpiB04, and bpiB07. While the BpiB07 protein was similar to a known lactonase, no significant similarities were observed for the BpiB01 and BpiB04 proteins or the deduced amino acid sequences. High-performance liquid chromatography–mass spectrometry analyses confirmed that the identified genes encode novel lactone-hydrolyzing enzymes. The original metagenome-derived clones were expressed in *Pseudomonas aeruginosa* and employed in motility and biofilm assays. All clones were able to reproducibly inhibit motility in *P. aeruginosa*. Furthermore, these genes clearly inhibited biofilm formation in *P. aeruginosa* when expressed in *P. aeruginosa* PAO1. Thus, this is the first study in which metagenome-derived proteins have been expressed in *P. aeruginosa* to successfully inhibit biofilm formation.

Quorum sensing (QS) is a cell density-dependent system of gene regulation in prokaryotes. Through the accumulation of bacterially produced signal molecules, autoinducers (AIs), the bacterial population is able to sense increases in cell density and then alters gene expression accordingly (50). This enables the coordinated expression of genes at the population level which are most effective at higher cell densities, such as those coding for pathogenicity and production of extracellular proteins (18, 45). Many QS mechanisms involve *N*-acyl-homoserine lactones (AHLs) in gram-negative bacteria and modified oligopeptides in gram-positive bacteria (50). Bacteria are also able to detect and respond to the number of unrelated species through an interspecies QS system involving furanosyl diesters that at least in some cases contain boron (autoinducer 2 [AI-2]). AI-2 activity has been detected in many different bacteria, and *luxS*, the gene involved in AI-2 synthesis, has been identified in more than 50 different bacteria (13).

As QS regulates expression of pathogenicity-related phenotypes, such as biofilm formation and virulence in many bacteria, including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli*, this is a potential target for antipathogen treatment (6, 32). There are many signal interference mechanisms called anti-QS mechanisms. They are distinguished as "quorum sensing inhibition" (QSI) for those that interfere with the QS gene regulation system and "quorum quenching" for those that degrade the AI molecules (33, 53). As the many levels of complexity of QS regulation are elucidated, possible new targets for anti-QS are revealed.

Many of the original investigations of QS relied on pure culture analysis for determination of the QS signals (11, 30, 31). In contrast, the search for novel quorum-quenching strategies can be achieved using cultivation-independent methods. Metagenomics enables investigations of the genetic potential present within the collective microbial complement of a habitat (17). This technique has been used to find many novel, biologically active molecules by our and many other labs (38). The relatively high number of bacteria in soil that are able to degrade AHLs (5) and the high genomic diversity of soil microorganisms (4) make it an ideal habitat for the application of metagenomics in the search for novel compounds involved in inhibition of QS. To date, only a very limited number of studies have employed metagenomics to identify novel anti-QS mechanisms (35, 51), and there is little information available on whether any of the identified genes and deduced proteins interferes with bacterial biofilm formation.

Therefore, we describe in this report the isolation and genetic characterization of three novel metagenome-derived lactonases displaying altered QS and motility phenotypes in *Agrobacterium tumefaciens* and *P. aeruginosa*. Furthermore, we describe biofilm phenotypes in *P. aeruginosa*, and we show that the phenotypes observed are probably the result of the AHL degradation through the metagenome-derived proteins.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* DH5α (Invitrogen, Karlsruhe, Germany), *E. coli* XL1-Blue (Stratagene, La Jolla, CA), and *P. aeruginosa* PAO1 were maintained in LB medium (36) at 37°C. For clones containing the phagemid vector pBK-CMV, kanamycin (final concentration of 25 μg ml^{-1}) was added, for clones containing the vector pBluescript SK+ (Stratagene, La Jolla, CA), ampicillin (100 μg ml^{-1}) was added, and for clones containing the broad-host-range vector pBBR1MCS-5 (21), gentamicin (10 or 50 μg ml^{-1})

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TABLE 1. Primers used for amplification and cloning of confirmed quorum-quenching ORFs

ORF	Primer	Primer sequence (5′ to 3′)	Product size (bp)
bpiB01	Forward	GGGGACTCGAGAAATGAAA AATTTGACC	1,231
	Reverse	TCGAGTCGAAGCTTTCAGA GCAGGAT	
bpiB04	Forward	GCAGGTACCATATCTCTCGT CATGGCGTTG	547
	Reverse	TTCACACAGGAAACAGCTA TGACC	
bpiB07	Forward	GTGCCGCTTTCACTCGAGCT CTTGATGGAT	698
	Reverse	ACGACGTTGTAAAACGACG GCCAG	

was added. The *E. coli* carrying the *celA* gene was derived by the method described in reference 48.

A. tumefaciens NTL4 (15), which carries a *traI-lacZ* promoter fusion, and *A. tumefaciens* KYC6 (16), which is a natural overproducer of homoserine lactones, were grown in LB or AT medium (43) containing 0.5% glucose per liter at 30°C. For *A. tumefaciens* NTL4, spectinomycin (final concentration, 50 μg ml^{-1}) and tetracycline (final concentration, 4.5 μg ml^{-1}) were added.

Biofilm experiments in flow chambers were done using a modified alginate-promoting medium (mAPM) (25) at pH 7.5. The composition of mAPM was as follows: 10 mM sodium gluconate, 10 mM KNO_3, 1 mM $MgSO_4$, 1.25 mM NaH_2PO_4, and 2.8 mM K_2HPO_4. ABt medium using glucose (final concentration, 1 mM) and glutamate (final concentration, 7.5 mM) as the carbon source (3) was also used in biofilm experiments.

Metagenomic library construction. Two metagenomic libraries were constructed from environmental soil samples collected from a field site next to the Department of Microbiology of the University of Göttingen. The DNA was extracted by following the protocols described previously (12, 39). The metagenomic DNA was cloned into the phagemid vector pBK-CMV using the ZAP Express cDNA synthesis kit (Stratagene, La Jolla, CA) and maintained in *E. coli* XL1-Blue. A total of 7,392 clones were generated with rather small insert sizes ranging between 2.5 and 6 kb.

Extraction of homoserine lactones from *A. tumefaciens* KYC6. *A. tumefaciens* KYC6, which overproduces *N*-(3-oxooctanoyl)-L-homoserine lactones (3-oxo-C_8-HSLs), was grown as 500-ml cultures in AT medium containing a double concentration of glucose for 3 days at 30°C (52). The produced 3-oxo-C_8-HSLs together with smaller fractions of other AHLs (i.e., 3-oxo-C_6-HSL) were extracted from the culture supernatant using 3 volumes of ethyl acetate, which were then evaporated using a rotary evaporator. The mixtures of AHLs obtained were dissolved in 1 ml ethyl acetate and stored at −20°C.

Screening for quorum sensing-inhibiting clones using the reporter strain *A. tumefaciens* NTL4, carrying a *traI-lacZ* promoter fusion. The metagenomic library was initially screened for clones capable of inactivating homoserine lactone signaling molecules or capable of blocking the corresponding AHL receptor/promoter in *A. tumefaciens*. The screen comprised the reporter strain *A. tume-*

TABLE 2. Constructs and control strains used in this study

Vector or construct	Characteristic(s)[a]	GenBank accession no.	Source
Metagenome-derived clones identified in this work			
pBio1-pBKCMV	pBK-CMV containing 3.5-kb insert	EF530726	
pBio7-pBKCMV	pBK-CMV containing 4.0-kb insert	EF530732	
pBio9-pBKCMV	pBK-CMV containing 1.7-kb insert	EF530734	
Plasmid vectors and constructs used for expression of the Bpi proteins in *E. coli* BL21 cells			
pET21a	*E. coli* six-His-tagged expression vector; Amp^r		Novagen, Germany
pQE30	*E. coli* six-His-tagged expression vector; Amp^r; pQE30		Qiagen, Hilden, Germany
pET19b	*E. coli* ten-His-tagged expression vector; Amp^r		Novagen, Germany
pET21a-*bpi01*	pET21a containing the bpiB01 ORF cloned into the NdeI+NotI restriction site		
pET19b-*bpi04*	pET19b containing the bpiB04 ORF cloned into the NdeI+NotI restriction site		
pQE30_*bpi07*_BH	pQE30 containing the bpiB07 ORF cloned into the BamHI and HindIII site		
Plasmid vectors and constructs used for expression of the Bpi proteins in *P. aeruginosa* motility and biofilm experiments			
pBBR1MCS-5	Broad-host-range vector (21)		
pB1H1-pBBR	pBBR1MCS-5 containing the bpiB01 ORF		
pB1H1TM-pBBR	Knockout control of pB1H1-pBBR containing the EZ::TN<TET-1> transposon in the bpiB01 ORF		
pB7mS-pBBR	pBBR1MCS-5 containing the bpiB04 ORF		
pB7mSTM-pBBR	Knockout control of pB7mS-pBBR containing the EZ::TN<TET-1> transposon in the bpiB04 ORF		
pB9N5-pBBR	pBBR1MCS-5 containing the bpiB07 ORF		
pB9N5TM-pBBR	Knockout control of pB9N5-pBBR containing the EZ::TN<TET-1> transposon in the bpiB07 ORF		
p007-pBBR	pBBR1-MCS5 containing 2-kb cellulase gene; experimental control		

[a] Amp^r, ampicillin resistant.

faciens NTL4, which carries a plasmid-based *traR* and *traI* gene with a *lacZ* fusion so that activation of the *traI* gene is associated with the production of β-galactosidase (*lacZ* gene), and the activity of β-galactosidase can be reported using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as the substrate for the assay. Alternatively, 2-nitrophenyl-β-D-galactopyranoside (ONPG) (Karl Roth GmbH, Karlsruhe, Germany) was used for liquid assays.

For the *traI* induction, 3-oxo-C_8-HSL extracted from *A. tumefaciens* KYC6, which is a natural 3-oxo-C_8-HSL overproducer, was used. For screening metagenome libraries, the *A. tumefaciens* NTL4 reporter strain was added to soft AT agar (AT medium solidified with 1% agar [BD Difco, Heidelberg, Germany]) to a final cell density of 10^7 cells ml^{-1}. Spectinomycin (final concentration, 60 μg ml^{-1}) and tetracycline (10 μg ml^{-1}) were also added. Four hundred microliters of agar was pipetted into each well of the 48-well plates and used the same day. The minimum amount of homoserine lactone required by the reporter strain for quorum sensing to occur (54) and the maximum amount of homoserine lactone inactivated during a 20-h incubation with the *E. coli* XL1-Blue host was determined in control experiments. In the control experiments, dilutions ranging from 10^{-1} to 10^{-4} of the extracted AHLs were incubated with the host strain for 20 h at 37°C. Five microliters of these diluted AHL mixes was pipetted onto the AT screening agar and then incubated overnight at 30°C. In this way, the 1,000-fold dilution of the produced extract was determined to be the threshold for unspecific degradation of the AHLs by the *E. coli* host. The chosen AHL concentration was fivefold higher than the concentration of signal molecule nonspecifically inactivated by *E. coli* XL1-Blue under the experimental conditions. Overnight cultures of the *E. coli* clones were incubated with AHL for 20 h at 37°C. Then, 5 μl of the supernatant was pipetted on the AT screening agar, and this was incubated at 30°C overnight. Development of a blue color indicated quorum sensing, and tests that remained colorless indicated possible quorum quenching.

ONPG tests using the *A. tumefaciens* NTL4 reporter strain. For the ONPG tests in liquid media, 5 μl of a 10^{-11} M solution of 3-oxo-C_8-HSL (Sigma-Aldrich, Heidelberg, Germany) was added to 100 μl crude cell extract or purified protein (see below for preparation) (10 μg ml^{-1}) and incubated at 30°C in 100 mM potassium phosphate buffer at pH 7.0. Following incubation for 2 h, this solution was added to 5 ml of a freshly grown *A. tumefaciens* NTL4 *traI-lacZ* fusion strain in AT medium; the cells were adjusted to 1×10^7 cells ml^{-1} prior to the test. After 17 h of incubation at 30°C, 1 ml cell suspension was mixed with 20 μl toluene and vortexed for 3 min. To 800 μl of this solution, 200 μl of the ONPG solution (4 mg ml^{-1} in Z buffer [composition per liter, 16.1 g $Na_2HPO_4 \cdot 7H_2O$, 5.5 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.246 g $MgSO_4 \cdot 7H_2O$, 2.7 ml β-mercaptoethanol; pH 7.0]) was added. After incubation for 20 min at room temperature, the absorbance was measured at 420 nm.

Genetic analysis of positive clones. The inserts of the positive clones were sequenced using automated sequencing technologies (MegaBACE 1000 system; Amersham Bioscience). Gaps were closed by primer walking. All potential open reading frames (ORFs) were analyzed using BlastX (NCBI). Sequences were deposited in GenBank. Accession numbers are provided at the end of Materials and Methods.

For the detection of the respective ORFs involved in QSI, the EZ::TN<TET-1> insertion kit (Epicentre Technologies Corp., Madison, WI) was used for in vitro transposon mutagenesis or subcloning was employed. The potential QS-inhibiting ORFs were amplified using the primers (forward and reverse primers) summarized in Table 1. Clones were assayed using the *A. tumefaciens* reporter strain described above and using the *P. aeruginosa* motility assays.

***P. aeruginosa* motility assays.** For the motility tests in *P. aeruginosa*, the potential ORFs were cloned into the broad-host-range vector pBBR1MCS-5. The potential QS-inhibiting ORFs were amplified using the primers (forward and reverse primers) given in Table 1 and transformed into *P. aeruginosa* PAO1. The resulting constructs are described in Table 2. *Pseudomonas* swarming agar contained M9 medium (36) without NH_4Cl and included 0.05% glutamic acid. This was solidified with 0.5% Bacto agar (BD Difco, Heidelberg, Germany). Swimming plates contained M9 medium with NH_4Cl but no glutamic acid and were solidified with 0.3% agar. For motility tests, 1×10^8 cells of an overnight *P. aeruginosa* PAO1 culture were applied on the middle of the agar plate. The plates were incubated at 37°C for 48 h. Complementation experiments to restore the wild-type motility phenotype were done using 0.3 μmol *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C_{12}-HSL) (Cayman Chemicals Ltd.) for each sample.

Cultivation of *P. aeruginosa* PAO1 biofilms in flow chambers. Biofilms were cultivated in two-channel flow cells constructed of V10A stainless steel. The individual channel dimensions were 3 mm by 8 mm by 54 mm. The substratum consisted of standard borosilicate glass coverslips (24 mm by 60 mm; thickness, 0.17 mm) that were fixed on the upper and lower side of the stainless steel flow chamber (total volume of 1.3 ml), using additive-free silicone glue. The assembled flow cells with Tygon tubing (inner diameter, 3.17 mm) attached to the outlets of each channel were sterilized by autoclaving at 121°C for 15 min. All experiments were performed at 30°C. Prior to inoculation, the flow chamber was rinsed with the medium for 5 h at a flow rate of 20 ml h^{-1}, using a multichannel Ismatec IPC-N peristaltic pump. For inoculation, bacteria from a 24-h culture on LB agar plates were washed once and then resuspended in mAPM or ABt medium; each channel of the flow cell was inoculated with 5 ml of the cell suspension adjusted to approximately 10^8 cells ml^{-1}. The biofilm test was done in the same medium. After the medium flow was arrested for 1 h, it was resumed

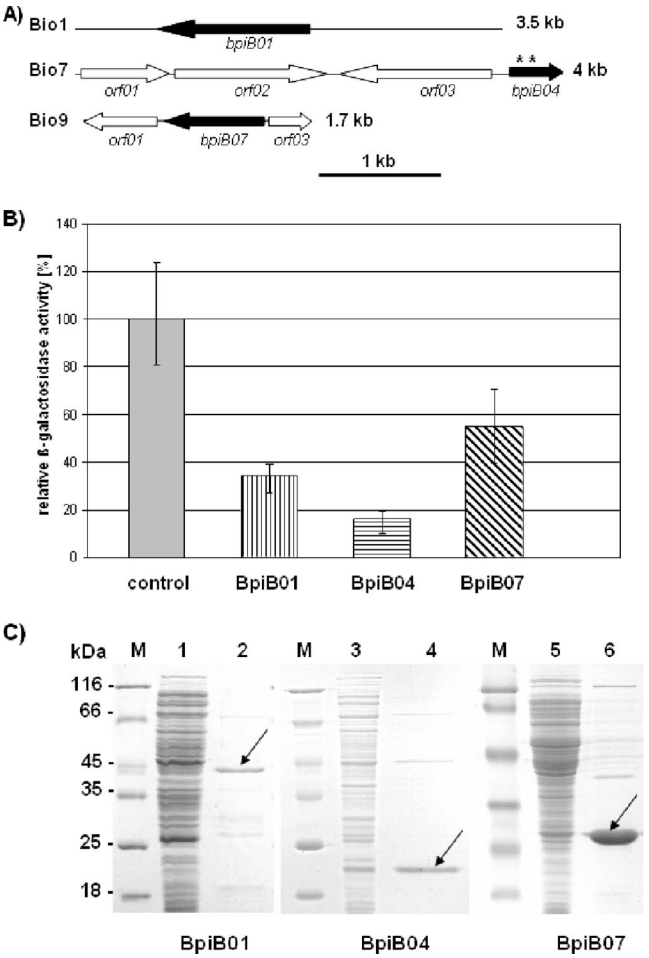


FIG. 1. (A) Arrangement of predicted ORFs on original metagenomic clones Bio1, Bio7, and Bio9, carrying the bpiB01, bpiB04, and bpiB07 genes. Black arrows indicate ORFs that were linked to the QSI phenotypes in *A. tumefaciens* and identified through subcloning or transposon mutagenesis. The locations of transposon insertions in the original metagenome clones are indicated by asterisks. DNA sequences of the corresponding clones have been deposited in GenBank. (B) Verification of AHL degradation using the *A. tumefaciens* reporter strain and protein extracts of *E. coli* cells overproducing the bpiB01, bpiB04, and bpiB07 genes. Tests were carried out in 1-ml cuvettes using ONPG as a substrate for the β-galactosidase. Data are mean values of at least three independent measurements. From left to right, control crude extract and crude extract from pET21a-*bpiB01*, pET19b-*bpiB04*, and pQE30-*bpi07* are shown by the bars in the graph. The tests contained equal amounts of protein (10 μg μl^{-1}) and 5 nM of AHLs (3-oxo-C_8-HSL). (C) Cell extracts of *E. coli* cells expressing Bpi proteins. The black arrows indicate the purified His-tagged protein. Lanes: M, marker proteins; 1, extract from cells expressing the bpiB01 gene; 2, purified BpiB01; 3, extract from cells expressing the bpiB04 gene; 4, purified BpiB04; 5, extract from cells expressing the bpiB07 gene; 6, purified BpiB07.

TABLE 3. Predicted BlastP analysis of biofilm phenotype-inhibiting ORFs

ORF	Size of protein (no. of amino acids)	Organism	Similarity (%)	Similarity (aa_{range}/aa_{total})[a]	Possible function	E value	GenBank accession no.
bpiB01	400	*Nitrobacter* sp. strain Nb-311A	57	25–383/384	Hypothetical protein	4e−71	ZP_01047427.1
bpiB04	135	*Pseudomonas fluorescens*	53	148–196/374	Acetylation of cellulose	4.9	AAL71849.1
bpiB07	265	*Xanthomonas campestris*	68	1–212/220	Putative diene-lactone hydrolase	8e−58	YP364619.1

[a] The range of similar amino acids (aa_{range}) to the total number of amino acids (aa_{total}) is shown.

at a rate of 20 ml h^{-1} (83.3 cm h^{-1}), corresponding to a flow with a Reynolds number of 0.73. The residual time of the bacteria in the flow chambers was approximately 4.5 min. Continuous monitoring of biofilm growth was performed using confocal laser scanning microscopy (CLSM) in the transmission mode and using the transmitted-light detector. After 72 h, biofilm cells were stained with SYTO 9 (Molecular Probes Inc., Eugene, OR) by injecting 5 ml of SYTO 9 solution (1.5 ml SYTO 9 ml^{-1} mAPM) and viewed by CLSM.

CLSM and image analysis of *P. aeruginosa* PAO1 biofilms in flow chambers. Visualization of flow cell biofilms was performed using an LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany). Images were obtained with a Zeiss LD Achroplan 40× objective with a numerical aperture of 0.60. The development of unstained biofilms in the flow cells was visualized by using the transmitted-light detector of the CLSM system, recording nonconfocal single-image slices. Three-dimensional image stacks of 72-h-old flow cell biofilms stained with SYTO 9 were recorded at an excitation wavelength of 488 nm by use of an argon laser in combination with an emission long-pass filter LP 505 nm. Digital image acquisition and analysis of the CLSM optical thin sections were performed with the Zeiss LSM software (version 3.2). Three-dimensional reconstructions were done with the Zeiss AxioVision software (version 3.1). Quantification of biofilm parameters (i.e., size, structure, and thickness) from the obtained image stacks (50 pictures with intervals of 2 μm) was performed using the COMSTAT program. Tests were verified in at least three independent experiments for each clone or their knockout mutant.

Transformation and conjugation procedures. Plasmid transformation in *E. coli* was done by following standard electroporation protocols and heat shock or conjugation protocols (36). For conjugation, the *E. coli* helper strain HBH101 bearing pRK2013 was employed (14). *P. aeruginosa* was transformed using electroporation (42).

Cell lysis and protein purification. For the preparation of the crude cell extracts, 200-ml LB cultures containing ampicillin (100 μg ml^{-1}) were grown at 30°C to an optical density at 600 nm (OD_{600}) of 1.0 to 1.5. Cells were harvested and resuspended in 1× LEW buffer (Macherey-Nagel, Düren, Germany) prior to cell disruption through sonication (UP 200S sonicator; Hielscher, Germany) at 50% amplitude and cycle 0.5 for 5 min or using a French pressure cell. After the cells were centrifuged at 13,000 rpm and 4°C for 30 min, the crude cell extract could be stored at −20°C until further use. The different Bpi proteins were then purified using Protino columns from Macherey-Nagel (Düren, Germany) by following the manufacturer's protocol. The levels of protein purity, as well as the molecular mass, were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

HPLC-MS analysis. For chemical analytics, 3-oxo-C_8-HSL (Sigma-Aldrich, Heidelberg, Germany) (21 μmol) (final concentration of 10.5 mM in a total volume of 1 ml) was mixed with either purified protein or crude extracts of *E. coli* cells overexpressing the bpi genes (20 μg ml^{-1}) in 100 mM potassium phosphate buffer (pH 7.0) and incubated for 20 h at 30°C. After incubation, the resulting mixtures were extracted twice with 1 volume of ethyl acetate, and the combined organic layers were concentrated in vacuo. For high-performance liquid chromatography (HPLC) analysis, each extract was dissolved in methanol (110 μl). HPLC-mass spectrometry-diode array detector (HPLC-MS-DAD) analyses of the solutions thus obtained were performed using a Grom Supersphere-100 RP-18 end-capped, 4-μm column (100 by 2 mm), a Flux instruments pump Rheos 4000, a PDA detector Finnigan Surveyor and mass spectrometer Finnigan LC-Q (70 eV) with software package Finnigan Xcalibur. A gradient program with solvent A (0.5% aqueous HCOOH) and solvent B (methanol) was used to detect the 3-oxo-C_8-HSL and the cleaved product (retention times of 8.3 min and 7.4 min, respectively): gradient from 20% solvent B to 100% solvent B in solvent A in 20 min, 10 min 100% solvent B to 20% solvent B in 2 min, 8 min 20% solvent B (total, 40 min program) at a flow rate of 300 μl min^{-1}. HPLC-MS-tandem mass analyses were recorded on a Finnigan LC-Q spectrometer (impact energy 25%), high-resolution mass spectra (electrospray ionization [ESI]) on a Bruker APEX IV 7T spectrometer; preselected ion peak matching at a resolution (R) of ≫10,000 was within 2 ppm of the exact masses. *N*-(3-oxooctanoyl)-L-homoserine was synthesized by chemical hydrolysis of the corresponding AHL (5.8 mg) in dimethyl sulfoxide (60 μl) with 1 N NaOH (1.5 equivalents, 36 μl) for 16 h at room temperature (8).

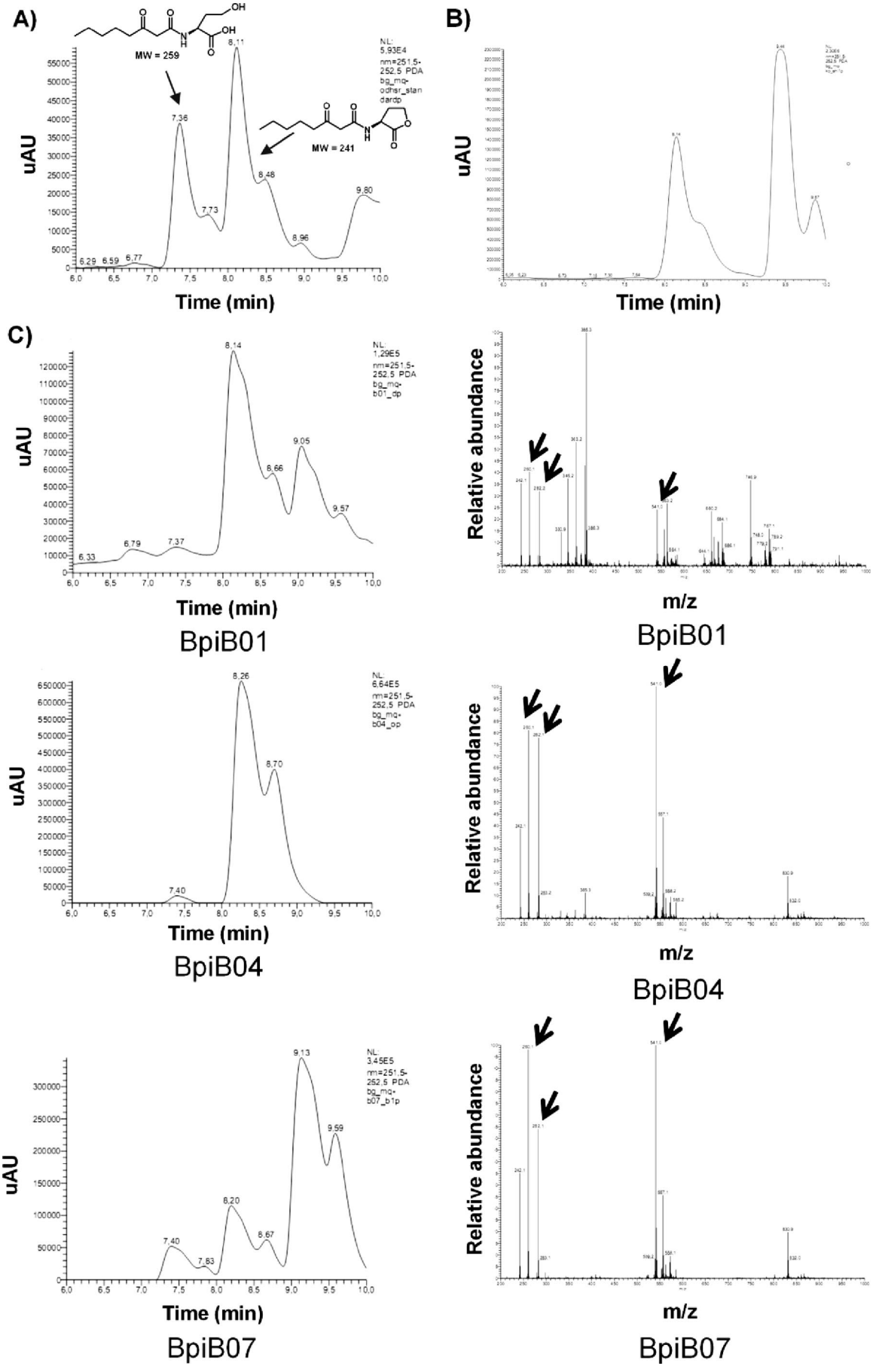
Measurement of the pyocyanin production in *P. aeruginosa* PAO1 cultures. Analysis of the pyocyanin production was done by measuring the absorbance of cell-free culture supernatants of *P. aeruginosa* PAO1 at 690 nm, the known maximum absorption of pyocyanin. The amount of produced pyocyanin could be calculated using the reported (26) extinction coefficient ε for pyocyanin at this wavelength (ε = 4,310 M^{-1} cm^{-1} at pH 7). For the complementation tests using 3-oxo-C_{12}-HSL (Cayman Chemicals Ltd.), 34 nM (bpiB01) or 68 nM (bpiB04 or bpiB07) was added to 5 ml of fresh culture. In addition, *N*-butyryl-DL-homoserine lactone (C_4-HSL) (Sigma-Aldrich, Heidelberg, Germany) was added at a 1.7 mM (bpiB01) or 2.3 mM (bpiB04 or bpiB07) concentration. These cultures were grown for 16 h at 37°C prior to the pyocyanin measurements.

Nucleotide sequence accession numbers. The sequences of the originally isolated clones have been deposited in GenBank under the following accession numbers: clone Bio1, EF530726; clone Bio7, EF530732; and clone Bio9, EF530734.

RESULTS

Detection of QS-interfering clones and genetic analysis. In the initial screen, a total of 7,392 metagenomic clones were tested three times using a screening system which included an *A. tumefaciens* reporter strain carrying a *traI-lacZ* reporter gene, X-Gal, and 3-oxo-C_8-HSL. A total of 438 clones consistently gave a positive result for QSI. At this stage, 50 of the clones were partially sequenced and compared with the NCBI database. The clones were all unique, and none of them showed identity to known quorum-quenching genes. All positive clones from the first screening were then subjected to a second screen including growth tests. Because clones Bio1, Bio7, and Bio9 reproducibly stayed colorless in the microtiter plates compared to the controls and most other clones, these clones were analyzed further.

The insert size of these clones ranged from 1.7 to 4.0 kb (Table 2 and Fig. 1). The inserts were completely sequenced, and potential ORFs were detected (Fig. 1A). Those ORFs initially shown to inactivate the AHLs during the first screen and also later proven to inhibit biofilm formation are labeled bpi for *b*iofilm *p*henotype-*i*nhibiting genes and are highlighted in Fig. 1. The observed similarities for the proteins were in general rather weak, with E values larger than e^{-80}. Thus, no definite conclusions on their functions could be drawn based on the BlastX analyses. The deduced amino acid sequence of Bpi07 revealed a low similarity to a putative dienelactone hydrolase from *Xanthomonas campestris*. BpiB07 is similar to the


A)
MW = 259
MW = 241
uAU
Time (min)
B)
uAU
Time (min)
C)
uAU
Time (min)
BpiB01
Relative abundance
m/z
BpiB01
uAU
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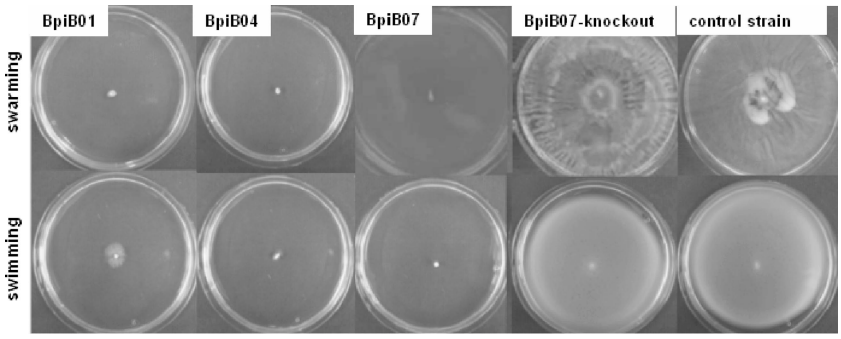

FIG. 3. Motility tests with *P. aeruginosa* PAO1 expressing the bpi genes. The top row shows the swarming phenotype, and the bottom row shows the swimming phenotype of the same clone. The pB1H1 subclone containing the bpiB01 gene in pBBR1MCS-5 (BpiB01), pB7mS subclone with the bpiB04 gene in pBBR1MCS-5 (BpiB04), B9N5 subclone containing the bpiB07 gene in pBluescript SK+ (BpiB07), control strain B9N5TM containing a transposon in the bpiB07 gene with the restored swarming phenotype in pBBR1MCS-5 (BpiB07-knockout), and experimental control pBBR1MCS-5 with a 2-kb cellulase gene (control strain) are shown.

esterase-lipase superfamily of proteins (E value of $2e^{-32}$) with significant similarities to the pfam 01738 domain of dienelactone hydrolase family proteins. BpiB04 was not similar to any known protein but reveals two GXXG motifs which are commonly found in glycosyl hydrolase family proteins. bpiB04 encoded the smallest protein consisting of only 135 amino acids. BpiB01 revealed no conserved domains in its amino acid sequence but was similar to a number of hypothetical proteins linked to recently finished genome projects. None of our proteins revealed a conserved domain known to be involved in lactonases in Zn^{2+} binding [GXXLXHE(H/A)XAXXXGX PXXH]. Similarities observed for the deduced proteins encoded by the ORFs bpi01, bpi04, and bpi07 are summarized in Table 3.

The metagenome-derived genes of bpiB01, bpiB04, and bpiB07 were amplified and cloned into an expression vector as described in Materials and Methods. The estimated molecular masses of the histidine-tagged and purified proteins of BpiB01, BpiB04, and BpiB07 (Fig. 1C) were in accordance with the theoretical molecular masses of 45 kDa (BpiB01), 18 kDa (BpiB04), and 29 kDa (BpiB07).

β-Galactosidase assays to verify the QS-inhibitory effects of the bpi genes. To further confirm that the identified clones indeed carried a gene that affected the quorum sensing response in the *A. tumefaciens* reporter strain NTL4 carrying a *traI-lacZ* promoter fusion, we cloned bpiB01, bpiB04, and bpiB07 into an expression vector (Table 2). The constructs were then tested for their influence on QS in *A. tumefaciens* (Fig. 1B). Tests using the *A. tumefaciens* strain NTL4 indicated a significant reduction of the AHLs in the presence of *E. coli* cell extracts carrying the bpi genes bpiB01, bpiB04, and bpiB07. In these tests, 5 nM of the 3-oxo-C_8-HSL was incubated together with crude cell extract of *E. coli* cells overexpressing the bpi genes. After 2 hours of incubation, the levels of detected AHLs were significantly lower than those of the controls. In general, less than 50% of the added AHLs could be detected (Fig. 1B). 3-Oxo-C_8-HSL degradation, however, was observed only in the presence of low concentrations of Zn^{2+} and Ca^{2+}, suggesting that all enzymes are probably metal-dependent metallohydrolases. In summary, these tests clearly indicate that bpiB01, bpiB04, and bpiB07 are involved in AHL degradation, and they confirmed the data obtained in the initial screen.

HPLC-MS analysis to verify the AHL degradation. To further test how the BpiB01, BpiB04, and BpiB07 clones inactivate the AHL signal, 3-oxo-C_8-HSL was incubated with either purified protein or crude extracts of *E. coli* cells overexpressing the bpi genes, and the reaction products were analyzed by HPLC-MS-DAD. Enzymatic degradation of 3-oxo-C_8-HSL resulted in a mixture consisting of 3-oxo-C_8-HSL and a more polar compound (retention times of 8.3 min and 7.4 min, respectively), as determined by HPLC analysis followed by ESI mass spectrometry (ESI-MS) (Fig. 2). ESI-MS analysis of the polar compound showed a $[M+H]^+$ ion at an *m/z* (mass-to-charge ratio) of 260.1, a $[M+Na]^+$ ion at an *m/z* of 282.1, and a $[2M+Na]^+$ ion at an *m/z* of 540.1 (Fig. 2). This mass increase of 18 is in agreement with a cleavage of a lactone ring of 3-oxo-C_8-HSL (molecular weight, 241) to yield the γ-hydroxy

FIG. 2. HPLC-MS analysis of 3-oxo-C_8-HSL after incubation with Bpi proteins. (A) Chemical controls show hydrolyzed lactone *N*-(3-oxooctanoyl)-L-homoserine (7.36 min) and the closed form (8.25 min) in the HPLC-UV chromatogram. The hydrolyzed 3-oxo-C_8-HSL was a synthetically produced AHL and purchased from Sigma Aldrich (Heidelberg, Germany). Spectra were recorded at 252 nm. For HPLC-MS-DAD analyses of chemical controls, see the supplemental material. uAU, microabsorption units; MW, molecular weight. (B) Further protein control experiment. 3-Oxo-C_8-HSL and 20 μg protein of extracts of *E. coli* BL21 cells that do not express any of the bpi genes were incubated with 21 μmol 3-oxo-C_8-HSL (final concentration of 10.5 mM in a total volume of 1 ml) for 20 h at 30°C. No peak was observed at 7.36 min in the HPLC-UV spectrum at 252 nm, and no significant $[M+H]^+$ ion at an *m/z* of 260 rather than a $[M+H]^+$ ion at an *m/z* of 282 was detected (data not shown). (C) Bpi proteins were incubated with 21 μmol 3-oxo-C_8-HSL (final concentration of 10.5 mM) for 20 h at 30°C. HPLC-MS-DAD analyses showed identical retention times for the hydrolyzed lactone ring of 3-oxo-C_8-HSL for both the synthetic and naturally derived compounds (left channel). All mass spectra show a $[M+H]^+$ ion at an *m/z* of 260.1, a $[M+Na]^+$ ion at an *m/z* of 282.1, and a $[2M+Na]^+$ ion at an *m/z* of 540.1 (right panel). These masses are indicated by black arrows. The tests were done with recombinant and purified proteins.

carboxylic acid. The substrate 3-oxo-C_8-HSL was detected in ESI-MS analysis by a $[M+H]^+$ ion at an *m/z* of 242.1 and a $[M+Na]^+$ ion at an *m/z* of 264.2 (Fig. 2). Tandem MS of the parent ion at an *m/z* of 260 showed the characteristic daughter ions of 242 and 120 resulting from the lactone-opened *N*-(3-oxooctanoyl)-L-homoserine (molecular weight, 259.1). Additionally, high-resolution MS of the $[M+H]^+$ ion at an *m/z* of 260.1 was consistent with the formula $C_{12}H_{21}NO_5$ of the γ-hydroxy carboxylic acid. For further confirmation, the 3-oxo-C_8-HSL was partially and fully hydrolyzed via alkaline hydrolysis to achieve the L-homoserine γ-hydroxy carboxylic acid from its corresponding lactone. HPLC-MS-DAD analysis showed that both the retention time and mass spectrum of the synthetic product were identical to those of the enzymatic degradation product (Fig. 2A). 3-Oxo-C_8-HSL incubated under the same conditions with *E. coli* cell extracts (Fig. 2B) but not expressing any of the bpi genes did not produce these peaks. Altogether, these data confirmed the lactonase activity of the clone bpiB04. The clones bpiB01 and bpiB07 showed the same enzymatic activity as clone bpiB04 did, and the chemical analysis of their degradation products of 3-oxo-C_8-HSL resemble the enzymatic products of bpiB04 (Fig. 2C) (see Fig. S1 and S2 in the supplemental material).

Again, the AHL degradation using purified proteins was observed only in the presence of low concentrations of Zn^{2+} (2 mM). Altogether, these data suggest that the three proteins analyzed in this work are probably novel lactonases and that their activities are metal dependent.

***P. aeruginosa* motility tests.** In *P. aeruginosa*, motility is QS dependent (20, 49). Therefore, the bpi ORFs were tested for their influence on motility in this microbe and the genes bpiB01, bpiB04, and bpiB07 were cloned into the broad-host-range vector pBBR1MCS-5. The resulting constructs were transformed into *P. aeruginosa* PAO1 (Table 2). The correctness of the inserts was verified by DNA sequencing (data not shown).

All clones strongly inhibited swarming on the swarming agar in comparison to the control, which consisted of a 2-kb control gene cloned into the same vector (Fig. 3). The level of inhibition was the same for all the clones. To further verify these results, knockout mutants were generated for the bpi genes (Table 2). These mutants displayed a restored wild-type swarming phenotype (Fig. 3). The use of a control strain carrying a previously characterized cellulose gene did not reveal a reduced motility (Fig. 3). Altogether, these controls confirmed that the motility phenotypes were indeed associated with the metagenome-derived DNA fragments. Adding small amounts of 3-oxo-C_{12} HSL could partially complement the inhibited swarming phenotypes (see Fig. S3 in the supplemental material).

Furthermore, it is known that pyocyanin production is QS dependent in *P. aeruginosa* (37). Therefore, we set out to test the effect of the metagenome-derived bpi genes on pyocyanin production. Again, all motility-inhibited clones showed significantly reduced levels of pyocyanin production in contrast to the controls where pyocyanin production was observed after 12 to 16 h growth in LB medium at 37°C on the shaker (Fig. 4). Also, the formation of cell aggregates was not observed for the motility-inhibited clones. However, the control, carrying the cellulose gene, as well as bpi-knockout control strains showed high levels of pyocyanin production (Fig. 4). Furthermore, complementation tests performed by adding C_4-HSL at 1.7 mM (bpiB01) or 2.3 mM (bpiB04 or bpiB07) together with 3-oxo-C_{12}-HSL at 34 nM (bpiB01) or 68 nM (bpiB04 or bpiB07) mostly restored the pyocyanin phenotypes (Fig. 4). In these tests, the clones carrying the bpi genes produced almost the same amounts of pyocyanin as measured in the control strains or the parent strain *P. aeruginosa* PAO1 (Fig. 4). This phenotype, however, was observed only when both 3-oxo-C_{12}-HSL and C_4-HSL were added to the cultures.

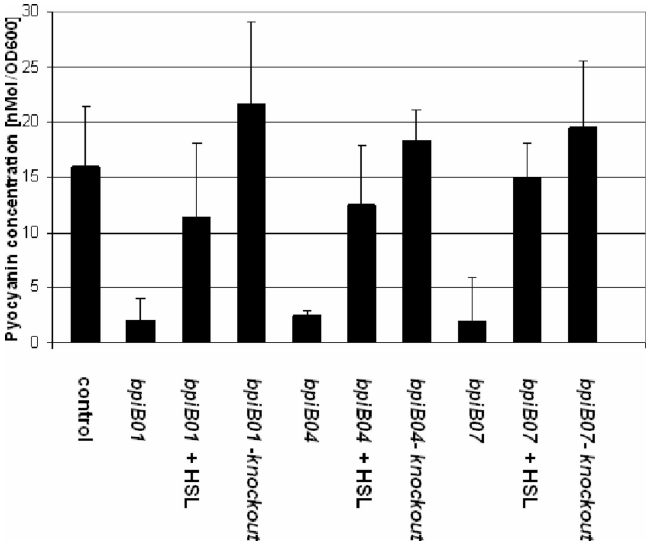

FIG. 4. Pyocyanin observed in *P. aeruginosa* carrying bpi genes in the presence and absence of added C_4-HSL and 3-oxo-C_{12}-HSL. From left to right, pyocanin levels (in nanomoles/OD_{600}) in experimental control pBBR1MCS-5 with a 2-kb cellulase gene (control), pB1H1 with the bpiB01 gene in pBBR1MCS-5 (*bpiB01*), pB1H1 with the bpiB01 gene in pBBR1MCS-5 with C_4-HSL and 3-oxo-C_{12}-HSL (*bpiB01* + HSL), pB1H1TM control strain containing a transposon in the bpiB01 gene (*bpiB01*-knockout), pB7mS with the bpiB04 gene in pBBR1MCS-5 (*bpi04*), pB7mS with bpiB04 in pBBR1MCS-5 with C_4-HSL and 3-oxo-C_{12}-HSL (*bpi04* + HSL), pB7mSTM control strain containing a transposon in the bpiB04 gene (*bpiB04*-knockout), pB9N5 with the bpiB07 gene in pBBR1MCS-5 (*bpiB07*), pB9N5 with the bpiB07 gene in pBBR1MCS-5 with C_4-HSL and 3-oxo-C_{12}-HSL (*bpiB07* + HSL), and pB9N5TM control strain containing a transposon in the bpiB07 gene (*bpiB07*-knockout) are shown. Tests were done at 37°C for 16 h, and C_4-HSL and 3-oxo-C_{12}-HSL were added at the same time and at 1.7 mM (bpiB01) or 2.3 mM (bpiB04 or bpiB07) C_4-HSL and 34 nM (bpiB01) or 68 nM (bpiB04 or bpiB07) 3-oxo-C_{12}-HSL concentrations. The cell densities of all cultures analyzed in these tests ranged from 2.2 to 2.4 at an OD_{600}. Data represent mean values of at least three independent cultures, and error bars indicate the standard deviations.

Additional growth experiments in liquid media, the mAPM medium, LB medium, and the ABt medium, showed that none of the bpi genes or any of the metagenome-derived fragments inhibited growth of *P. aeruginosa* during exponential growth. The generation times were similar for the wild type and clones containing bpi genes. The main difference was that all the clones entered stationary phase earlier and did not reach as high a cell density, with OD_{600}s of 2.9 to 3.7 compared to an OD_{600} of 4.1 for the control containing the vector plus a 2-kb cellulase insert.

***P. aeruginosa* PAO1 biofilm tests.** To further analyze the role of the metagenome-derived clones, biofilm tests were conducted with *P. aeruginosa* using the Bpi clones and the corresponding control strains (Table 2). After 72 h, the control strain, carrying the cellulase gene in the same vector, had

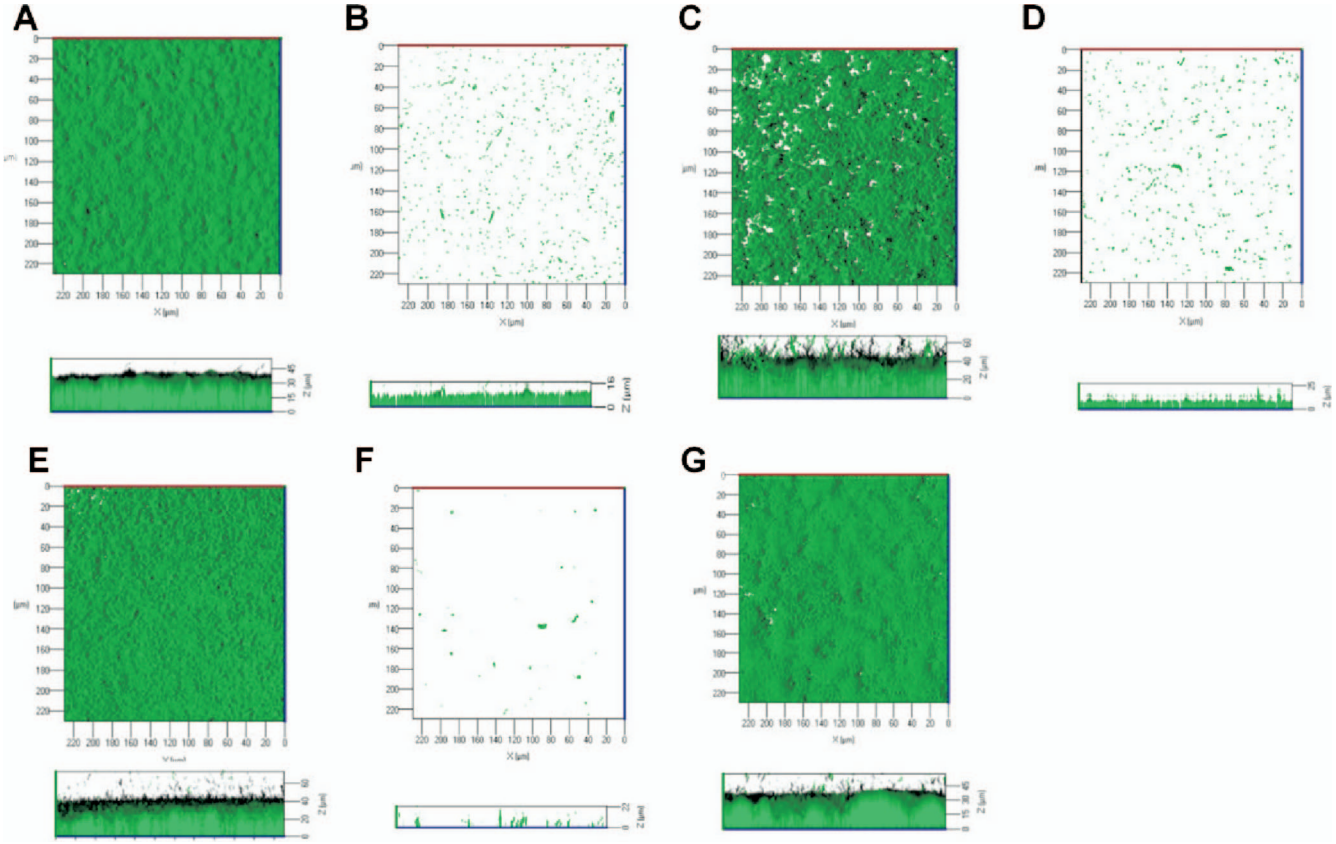

FIG. 5. Biofilm phenotypes of the *P. aeruginosa* PAO1 carrying metagenome-derived bpi genes after staining with SYTO 9 after 72 h. (A) Experimental control pBBR1MCS-5 with a 2-kb cellulase gene; (B) pB1H1 subclone containing the bpiB01 gene in pBBR1MCS-5; (C) pB1H1TM control strain containing a transposon in the bpiB01 gene; (D) subclone pB7mS with the bpiB04 gene in pBBR1MCS-5; (E) pB7mSTM control strain containing a transposon in the bpiB04 gene; (F) subclone pB9N5 containing the bpiB07 gene in pBBR1MCS-5; (G) pB9N5TM control strain containing a transposon in the bpiB07 gene.

formed thick uniform biofilms with a thickness of 35 to 40 μm (Fig. 5A). At the same time, all the bpi genes tested caused formation of poorly developed biofilms which had not progressed beyond attachment to the surface (Fig. 5B, D, and F). This indicates that all the bpi genes inhibit biofilm formation in *P. aeruginosa* at a very early stage, prior to microcolony formation. The strongest level of inhibition was displayed by bpiB07 (Fig. 5F), and bpiB01 displayed the lowest level of inhibition (Fig. 5B). Again, the additional control strains of the bpi genes, carrying a transposon in the respective gene, all displayed an almost fully restored biofilm formation phenotype (Fig. 5C, E, and G). This confirmed our observation that the phenotypes were linked to the respective bpi genes. These results were obtained for two different media, the mAPM medium and the ABt medium. Therefore, the phenotypes observed were not influenced or dependent on the growth medium used in these experiments.

DISCUSSION

In this study we have isolated and genetically characterized three metagenome-derived clones for their effects on bacterial motility and biofilm formation in *P. aeruginosa*. The metagenome-derived clones were initially identified because they repeatedly interfered with quorum sensing in an *A. tumefaciens*-based bioassay using a *traI-lacZ* reporter fusion. The genes identified and linked to the QSI phenotypes were designated bpiB01, bpiB04, and bpiB07. The bpi genes, when expressed in *P. aeruginosa*, resulted in the observation of motility and biofilm phenotypes. Despite controversy on whether quorum sensing is involved in *P. aeruginosa* motility (1), it is now generally accepted that quorum sensing influences motility and biofilm formation in *P. aeruginosa* at different levels of gene expression (40, 47, 49). With respect to the reduced biofilm formation and motility induced through the expression of the bpi genes, we hypothesize that these phenotypes are mainly a result of AHL degradation caused by the expression of the Bpi proteins in the *P. aeruginosa* cells. In fact, the results presented in Fig. 1B strongly supported that hypothesis, and data using more sophisticated analytical technologies (i.e., HPLC-MS) confirmed this hypothesis (Fig. 2).

Furthermore, our results are in line with the reports on enzymatic degradation of AHLs. Enzymatic degradation of the quorum sensing signal molecules is an established method of quorum quenching and has been reviewed very recently (32). Most bacterial AHL-specific lactonases known today reveal a conserved zinc binding domain HXHXDH motif and all the reported lactonases are hydrolases (EC 3). Also, most enzymes

are usually not very specific in their action with respect to the length of the acyl side chain attached to the lactone ring. Altogether not more than five different clusters (families) of AHL lactonases are known, all of them have been identified within the last 8 years (10, 32). Among the known enzymes involved in enzymatic degradation, lactonases and acylases are the best known examples. Both enzymes have been identified in a range of gram-negative and gram-positive microbes. Lactonases have been reported for *Bacillus* (7, 23, 44) *Agrobacterium* (2), *Rhodococcus* (28), *Streptomyces* (29), *Arthrobacter* (27), *Pseudomonas* (41), and *Klebsiella* (27) species. Acylases have been identified in *P. aeruginosa* (19), *Ralstonia* (24), and *Streptomyces* (29). While lactonases hydrolyze the lactone ring in a reversible way, the aminoacylases cleave the lactone ring off the fatty acids. Furthermore, it is also known that *P. aeruginosa* and *Variovorax paradoxus* are capable to grow on AHLs as the sole carbon and nitrogen source (22). Although BpiB07 is similar to a lactonase, no significant similarities were observed for any of the two other bpi genes or deduced amino acid sequences. Furthermore, Uroz et al. have recently reported a novel class of lactonases derived from *Rhodococcus*. These novel genes were designated *qsdA* and form a new protein family within the metal-dependent lactonases (46). However, the QsdA proteins are not similar to any of our Bpi proteins. Our metagenome-derived proteins are also different from the quorum-quenching clone recently reported by Riaz et al. which was also derived from a metagenome (35). Thus, with the exception of bpiB07, our bpiB01 and bpiB04 genes represent novel examples of AHL-degrading genes and further extend the diversity of the lactonase family proteins.

Concerning a potential application of our proteins for the prevention of microbial biofilms, at this time, we can only speculate about the success of such an attempt. However, taking into account the strong phenotypes observed in our motility and biofilm tests (Fig. 3 and 5), it might indeed be possible to use these proteins to quench the QS signal and thereby suppress bacterial biofilm formation at a very early stage. In fact, several examples demonstrating that the expression of quorum-quenching enzymes can result in the reduction of pathogenicity and virulence have been published (9, 24, 34, 35). However, we believe that this strategy might be more effective if the Bpi proteins were applied exogenously to developing biofilms or immobilized onto surfaces. In addition, the Bpi proteins would have to be encapsulated to protect them from proteases and other hydrolytic activities. Thus, future work will have to assess the feasibility of this approach and focus on the influence of the Bpi proteins on biofilm formation once they are added exogenously to developing *P. aeruginosa* or mixed-species microbial biofilms.

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